The criteria of measurement of the inorganic acid test of pollen viability

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The application of sulphuric acid to pollen causes the formation of protrusions of the vegetative cell from the germinative pores of pollen grains, and in aperturate pollen these protrusions resemble true pollen tubes. In this study, the sulphuric acid-induced pseudo pollen tubes of *Petunia* were examined to determine the criteria of measurement of this pollen viability testing procedure. The inorganic acid test of pollen viability does not differentiate between living and dead pollen grains, as was reported earlier, but measures the presence of the vegetative cell, bounded by an intact cell membrane. The procedure requires only low-magnification microscopy and takes a few seconds to perform. It is a simple, rapid, indirect microscopic test for the estimation of pollen viability in fresh pollen under field conditions. This procedure facilitates the obtaining of naked male gametophytes, and further development of the method may assist in the study of organelles and the internal structure of the pollen grain.

Swaelsuurbehandelings van stuifmeel lei tot die vorming van uitstulpings van die vegetatiewe sel by die kiemopeninge en in aperturaat-stuifmeelkorrels toon hierdie uitstulpings 'n morfologiese ooreenkoms met ware stuifmeelbuise. Swaelsuurgeïnduseerde pseudo-stuifmeelbuise is elektronmikroskopies ondersoek om die kriteria van meting van hierdie anorganiese suur-stuifmeeltoets te bepaal. Die toets onderskei nie tussen lewende en dooie stuifmeelkorrels soos vroeër gerapporteer nie, maar meet wel die teenwoordigheid van die vegetatiewe sel, gebind deur 'n intakte selmembraan. Die prosedure word binne enkele minute voltooi en kan teen lae vergroting waargeneem word. Dit bied dus 'n eenvoudige, maklike, indirekte metode om stuifmeelkwaliteit onder veldtoestande te skat. Hierdie metode vir die verkryging van naakte manlike gametofiete kan met verdere ontwikkeling moontlikhede inhou vir die studie van organelle en die interne struktuur van die stuifmeelkorrel.

Keywords: Inorganic acid test, *Petunia*, pollen protoplast, pollen viability.

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Introduction

Determination of pollen viability in fresh and stored pollen samples is fundamental to all plant-breeding programmes. There is no universal test for pollen viability, and the choice of testing procedure is dependent on factors such as pollen type, and cost and duration of the testing procedure. In plant breeding, a simple microscopic test to determine pollen viability in fresh pollen under field conditions is often required. Many procedures to determine the viability of the male gametophyte have been described (reviewed by Shivanna & Johri 1985; Shivanna & Rangaswamy 1992). The ultimate test of pollen viability is direct measurement of the ability of the pollen grain to effect fertilization (Shivanna & Rangaswamy 1992), but fruit and seed set determinations are costly, cumbersome and difficult to quantify (Shivanna & Johri 1985; Knox 1984; Stanley & Linskens 1974). Furthermore, seed set determinations may be invalid in the case of self-incompatibility systems or where the female parent used for the procedure is not highly fertile (Stanley & Linskens 1974; Janssen & Hermsen 1976).

Several indirect pollen-testing procedures are available to plant breeders to test pollen quality prior to field pollinations, although most of these tests are criticized for over-estimation of pollen quality (Heslop-Harrison *et al.* 1984; Knox 1984; Shivanna *et al.* 1991a). Indirect tests do not take into account the loss of pollen vigour as a result of storage or exposure to stress conditions such as heat and temperature (Shivanna *et al.* 1991a, b), but these procedures provide indices for the quality of pollen in a sample. Indirect testing procedures are particularly useful in the screening of fresh pollen. These tests generally measure the presence of one or more criteria associated with living pollen grains or living plant cells.

In germination testing, the ability of the pollen grain to germinate, and growth of the pollen tube is measured on an appropriate stigma or in artificial culture media. Although germination tests are routinely performed for pollen from several species, optimal germination conditions for pollen from many economically important crops have not been established and it has also been shown that pollen may germinate *in vitro*, but prove worthless in pollination experiments (Shivanna & Johri 1985; Knox 1984).

Non-germination tests measure qualities associated with living plant cells. These procedures may involve simple histochemical testing which measures the presence of cytoplasm, or sophisticated procedures combining different fluorochromes to investigate the state of the plasma membranes, enzyme activity and nuclear state of individual pollen grains (Heslop-Harrison & Heslop-Harrison 1970; Alexander 1980; Coleman & Goff 1985; Greissl 1989).

One method, proposed as a test of pollen viability, involves the application of inorganic acids to pollen (reviewed by Stanley & Linskens 1974; Shivanna & Johri 1985). Contrary to earlier reports, we have found that the conditions for the procedure can be standardized for the apertured pollen of *Chrysanthemum* (unpublished results). We investigated the reaction of pollen grains of several taxa, including diverse pollen types such as *Panicum, Datura, Ipomoea* and *Canna* to sulphuric acid and found that the response of pollen grains to inorganic acids is influenced by (i) the concentration of the acid, (ii) the relative humidity during storage, (iii) maturity of the pollen grain, (iv) the number of germinal apertures, (v) the morphology of the germinal apertures, and (vi) the genetic properties of the donor plant. In general, low concentrations of sulphuric acid cause bursting of pollen grains, whereas higher concentrations cause

release of the naked pollen protoplast in inaperturate and mono- and tri-aperturate pollen grains. At specific intermediate concentrations, the vegetative cell of aperturate pollen is observed to protrude from the germinal apertures, i.e. pseudo pollen tubes. The concentration of sulphuric acid required to induce any one of the above reactions is species specific, and the shape of the protrusion is influenced by the morphology of the exitus (unpublished results).

Bursting of pollen grains may be induced with hydrochloric acid in pollen grains of several angiosperm species (Koul & Paliwal 1961). Both sterile male pollen and pollen known to be unviable fail to burst upon treatment with inorganic acids. Upon bursting, cytoplasmic threads are formed when the ejected cytoplasm coagulates in the acid medium, and these threads are unwalled (Koul & Paliwal 1961). Quantification of pollen viability using the bursting technique is practically impossible, as the long threads of ejected cytoplasm become enmeshed and in some instances these threads become detached from the pollen grains from which they were ejected (unpublished results).

Earlier workers proposed that the pre-treatment of pollen in germination medium prior to the addition of sulphuric acid to the pollen suspension induced resumption of metabolic activity and synthesis of intine materials. Hence, the formation of so called 'instant pollen tubes', bounded by a normal pollen tube wall, were reported to be limited to live pollen only (Linskens & Mulleneers 1967). Although pre-treatment in germination media is recommended (Linskens & Mulleneers 1967; Stanley & Linskens 1974) we found that pre-treatment is not a prerequisite for the formation of these artificial pollen tubes. The criteria for measurement of the inorganic acid treatment of pollen to induce the formation of these pseudo pollen tubes, and the mechanism of tube formation in pollen testing procedures have not been resolved.

In this article we attempt to determine the criteria of measurement of the inorganic acid test for pollen viability. The likelihood of a normal pollen tube wall surrounding the pseudo pollen tubes formed by fresh *Petunia* pollen, without pre-treatment in germination medium, upon contact with sulphuric acid, will be examined. The effect of pre-hydration prior to acid application on the formation of these artificial pollen tubes is also explored. To obtain more definitive information about the mechanism of pseudo tube formation, the ultrastructural aspects of sulphuric acid-induced pseudo pollen tubes were investigated.

Materials and Methods

Plants of *Petunia* F_1 hybrids were purchased from a local nursery (Pretoria) and maintained under natural lighting regimes and at ambient autumn temperatures in the greenhouse. Fresh pollen was harvested from mature, dehiscing anthers by floating the anthers on appropriate concentrations of sulphuric acid and agitation with a glass rod, or by shaking the freshly dehisced anthers over a glass petri dish.

To determine the effect of pre-hydration prior to acid treatment, aliquots of *Petunia* pollen were harvested in glass vials. Fresh as well as heat-killed pollen samples were pre-hydrated in 0.5 M sucrose for 20 min at room temperature. Heat treatment was applied by subjecting a sample of the harvested pollen to heat (70°C) for 2 h. All samples subjected to these pre-treatments were allowed to reach room temperature prior to acid application (0 to 80% sulphuric acid, 5% increments). These procedures were repeated at least twice and 200 to 300 pollen grains were studied in each replicate.

To obtain *in vitro*-germinated pollen tubes, 5 mg of fresh pollen was germinated in 1 ml germination medium containing 10% sucrose and 0.01% boric acid (pH 6.5) for 3 h at 26°C. The inorganic acid test was applied directly to fresh *Petunia* pollen on a microscope slide. For pre-treated pollen samples, sulphuric acid was added to the pollen suspensions to the final concentration required.

For the inorganic acid treatment of electron microscopy samples, 5 mg of pollen was treated with 100 µm of 25% sulphuric acid (Merck 95–97%) for 60 sec. Samples were immediately diluted with 0.075 M sodium phosphate buffer, pH 7.4–7.6 and centriftiged for 1 min at 500 g. The supernatant was decanted and pollen was subjected to three further washes in sodium phosphate buffer. *In vitro*-germinated pollen tube samples, pseudo pollen tube samples, as well as untreated, mature pollen grains were fixed in 2.5% gluteraldehyde in the above buffer for 1 h followed by post-fixation in aqueous osmium tetroxide for 30 min. Acetone dehydration was followed by infiltration and embedding in Quetol 651 epoxy resin and the samples were polymerized at 60°C for 48 h (van der Merwe & Coetzee 1992). Sections were contrasted with aqueous uranyl acetate and lead citrate (Reynolds 1963). Ultra-thin sections, 70–90 nm thick, were viewed with a Philips EM 301 transmission electron microscope.

Results and Discussion

The terminology applied to the inorganic acid treatment of pollen requires clarification. Upon bursting, cytoplasm oozes from the pollen grains. These unwalled cytoplasmic exudates have been termed artificial pollen tubes (Koul & Paliwal 1961) or cytoplasmic threads (Linskens & Mulleneers 1967). The formation of inorganic acid-induced walled structures at the germinal apertures have been referred to as instant pollen tubes (Linskens & Mulleneers 1967). We propose that the walled protrusions formed at the germinal apertures after treatment with sulphuric acid represent an intermediate stage in the acid-induced liberation of the pollen protoplast, and the results of this study confirm that these protrusions are not depended on metabolic activity or pollen tube growth. The terms pseudo pollen tube (PPT) and cytoplasmic thread provide an unambiguous description of the walled and unwalled structures, respectively, formed by pollen on treatment with inorganic acids.

The optimal sulphuric acid concentration required for the formation of PPTs in pollen grains of *Petunia* was determined empirically by suspending fresh pollen in sulphuric acid concentrations of 5 to 45% (5% increments). While the application of 25% sulphuric acid leads to the formation of PPTs, liberation of the pollen protoplast was achieved with 35% sulphuric acid (Table 1). Bright field microscopic evaluation of these preparations showed that the tricolporate pollen of *Petunia* formed PPTs of varying length from one, two or three germinal apertures. These tubes are similar to true pollen tubes generated in culture, but in some of the tubes, bifurcation of the tube tip was observed.

Linskens and Mulleneers (1967) proposed that the pre-treat-

Table 1 Concentrations of sulphuric acid required to induce protrusions of the vegetative cell, liberation of the pollen protoplast and destruction of the pollen grain is altered by pre-treatment in sucrose solution in *Petunia*.

State of pollen		% H_2SO_4 required for producing different structures		
		Walled protrusions	Protoplast liberation	Destruction of pollen grain
Mature fresh or stored pollen grains	Without pre-hydration	25	35	>70
	With pre-hydration	5	-	>20
Heat-killed pollen grains	Without pre-hydration	25	35	>70
	With pre-hydration	-	-	>20

ment of the resting pollen grains in germination media resulted in resumption of enzyme activity and synthesis of intine material. This pre-treatment of pollen was reported to be a prerequisite for the formation of these walled structures, as the length of pre-treatment influenced the length of the tubes. In this study, protrusions of varying lengths were observed without pre-treatment in germination media, indicating that the length of the protrusion is not influenced by the availability of intine material. Pre-hydration of fresh pollen in sucrose solution greatly influences the reaction of pollen to sulphuric acid. The effect of pre-hydration of pollen grains on the concentrations of sulphuric acid required to induce vegetative cell protrusions, liberated protoplasts and destruction of pollen grains, in fresh and heat-killed pollen, are summarized in Table 1.

The optimal acid concentration required for partial extrusion of the pollen protoplast was five times lower in pre-hydrated pollen than in desiccated pollen. The PPTs formed in 25% sulphuric acid, without pre-treatment in germination medium, resemble true pollen tubes, but they often appear less regular in form. Pollen pre-treated in sucrose solution prior to the 5% sulphuric acid treatment elicits almost an identical reaction as 25% sulphuric acid applied to desiccated pollen. The only difference observed at low magnification, was that the PPTs emerging from fresh, untreated pollen show greater variation in length than those formed in pre-hydrated samples.

Pre-treatment of pollen in sucrose solution led to an overall lowering of resistance of the pollen grains to sulphuric acid. In pre-hydrated samples, the instantaneous destruction of pollen could be observed in 20% sulphuric acid and higher, as was reported earlier (Linskens and Mulleneers 1967). Instantaneous destruction of fresh pollen is evident in 70% sulphuric acid, whereas many grains remained intact for several minutes in undiluted sulphuric acid.

The application of 35% sulphuric acid to fresh pollen leads to the liberation of the pollen protoplasts via the germinal apertures. These protoplasts appear roughly triangular in shape, indicating coagulation of the peripheral cytoplasm had taken place at the germinal apertures, prior to extrusion of the protoplast. Liberation of the protoplast could not be achieved in pre-hydrated pollen samples at any concentration of sulphuric acid.

The response of pollen to heat treatment was influenced by the manner in which it was applied. Where the desiccated grains were placed in closed vials during the heat treatment (dry heat), followed by acid treatment, tube formation and protoplast liberation was achieved at the same concentrations required for untreated, desiccated pollen. However, heat treatment of pollen while suspended in sucrose solution (wet heat) and followed by acid treatment prevented both the formation of protrusions of the vegetative cell and liberation of the pollen protoplast.

The generally lower resistance of pollen grains after pre-hydration in sucrose solution and the fact that protoplast release could not be accomplished in pre-hydrated samples, suggest that pre-treatment of pollen in sucrose solution alters a quality of the pollen grain which affects the response of pollen to sulphuric acid. To determine whether the exine is altered by sucrose treatment, pollen samples were pre-hydrated in a sucrose solution and the suspension was left uncovered at 70°C for 2 h, allowing evaporation of the water prior to acid treatment. The exine of these grains ruptured and a spheroidal protoplast was released from the shattered exine. The spheroidal shape of the protoplast indicates that coagulation of the cytoplasm occurred symmetrically across the entire surface of the pollen grain.

Comparative transmission electron microscopic examination of fresh untreated pollen, sulphuric acid-treated pollen and *in vitro*-germinated pollen revealed striking ultrastructural differences between PPTs and true pollen tubes, with respect to the state of the cytoplasm and the tube walls.

Electron micrographs of the acid-induced PPTs in *Petunia* indicate that the cytoplasm is affected by the destructive action of the acid. However, the coagulated cytoplasm of the PPTs is electron opaque (Figure 1A) and similar to that of the protoplast of freshly dehisced, untreated pollen (Figure 1B). In the germinating pollen grains and true pollen tubes, the cytoplasm appears more electronlucent (Figure 1C). While the cytoplasm of *in vitro*-germinated grains is rich in rough endoplasmic reticulum (RER), as described by Herrero & Dickenson (1981), RER was seldom observed in the cytoplasm of grains with PPTs or in resting, untreated pollen grains. These observations provide final proof that the formation of inorganic acid-induced artificial pollen tubes are not dependent on reactivation of the pollen protoplast, as was proposed earlier (Linskens & Mulleneers 1967).

The walls of PPTs are not similar to *in vitro*-produced pollen tube walls. Short protrusions may be bounded by intine (Figure 2A), but thinning or absence of the intine was observed in the larger protrusions (Figure 2B). The intine appears to stretch and disintegrate as the PPT increases in length. In contrast, the intine of normal pollen tubes generated *in vitro* is continuous (Figure 1C). Vesicle formation of the cell membrane was observed in PPTs where the intine was absent (Figure 2C). PPTs are often typically bifurcated at the tip (Figure 3A, B).

The TEM study confirms that sulphuric acid-induced PPTs from untreated pollen grains are identical to the sulphuric acid-induced 'instant' pollen tubes produced by fully hydrated pollen, as described by Linskens & Mulleneers (1967). These workers proposed a bipartite hypothesis to explain the mechanism of the formation of these acid-induced tube-like structures. According to this hypothesis, the pre-treatment of pollen in germination medium results in the reactivation of the metabolism of the vegetative cell and the synthesis of intine materials. Subsequent inorganic acid treatment leads to rapid hydration of the vegetative cell, and the intine, which is also weakened by the pre-treatment, becomes more flexible to accommodate this increase in volume. The protrusions are formed at the sites of least resistance, i.e. the germinal apertures. This hypothesis does not clarify the formation of protrusions by desiccated pollen, nor does it provide an explanation for liberation of the naked pollen protoplast in concentrated sulphuric acid. The phenomena of bursting and PPT formation at different concentrations of inorganic acids are not accommodated by this hypothesis. Lastly, dead pollen grains respond to inorganic acid treatment in a similar way to live pollen grains, provided they are not subjected to a pre-soak in germination medium.

The results obtained in this study indicate that the mechanism of sulphuric acid-induced PPT formation is histochemically controlled. When pollen grains are placed in sulphuric acid, two reactions are to be considered. Firstly, pollen grains with an intact cell membrane hydrate at the low pH, as proposed by Linskens & Mulleneers (1967). Secondly, coagulation of the cytoplasm takes place in the acid medium, as shown by Koul & Paliwal (1961). All three responses, i.e. bursting, PPT formation and liberation of the pollen protoplast are explained if one considers that the reactions mentioned above occur at different rates in different acid concentrations.

At low concentrations of acid, coagulation of the pollen cytoplasm is ineffectual and the pollen grain swells in the acid medium until it explodes. This can be observed, with the ejection of unwalled, cytoplasmic threads. At higher concentrations, coagulation of the pollen cytoplasm occurs peripherally at the exposed area of the vegetative cell, i.e. the germinal aperture, resulting in a rigid wall which prevents the explosion of the grain. Swelling of the grain continues until the pollen cytoplasm is completely fixed or the cell membrane becomes impaired due

to the action of the acid. This results in the formation of PPTs. At even higher concentrations of acid, swelling occurs very rapidly and the pollen protoplast is expelled from its protective capsule. Coagulation of the cytoplasm takes place chiefly after liberation of the protoplast.

Where the surface of the pollen grain is protected by a func-

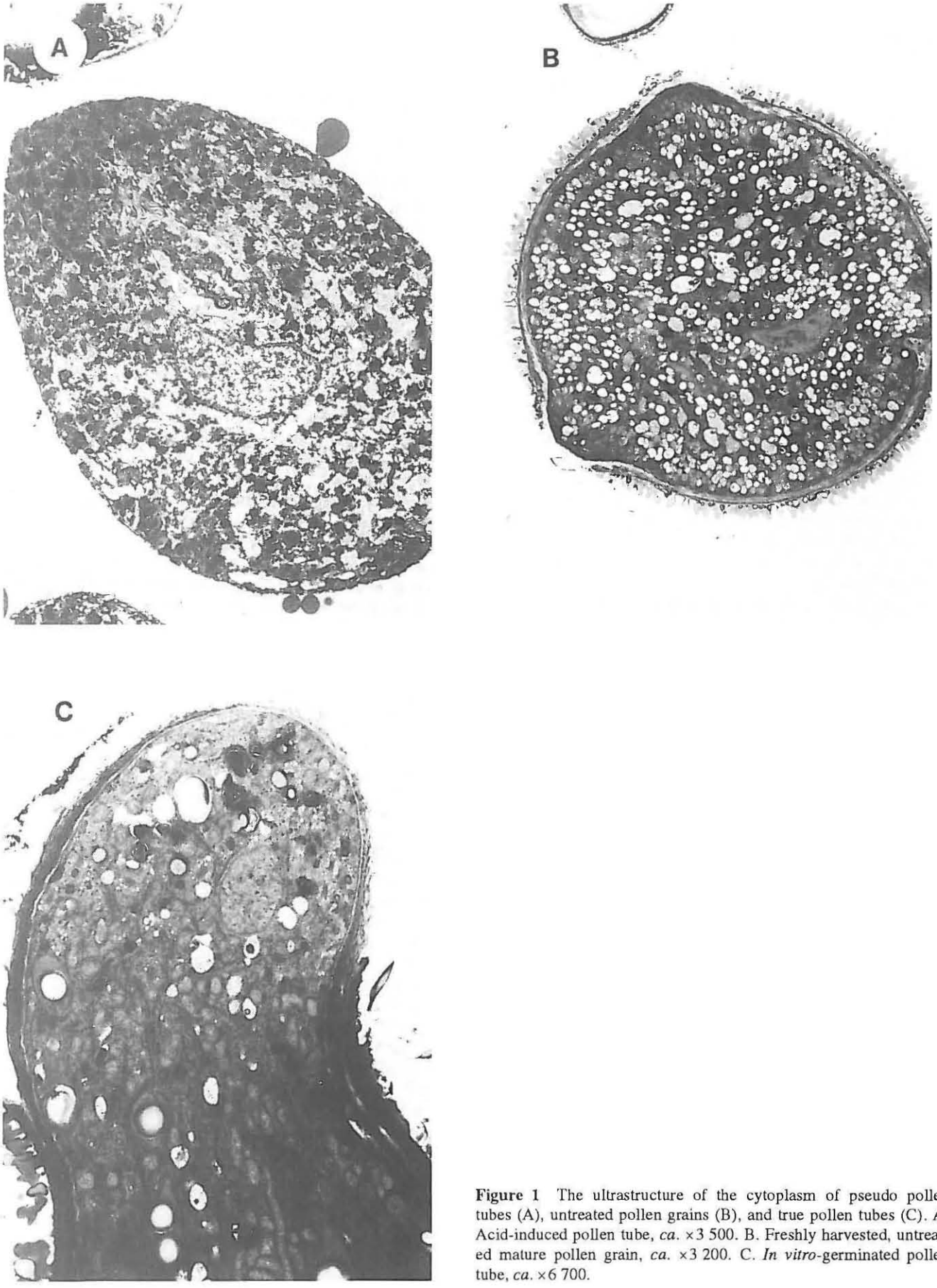

Figure 1 The ultrastructure of the cytoplasm of pseudo pollen tubes (A), untreated pollen grains (B), and true pollen tubes (C). A. Acid-induced pollen tube, *ca.* ×3 500. B. Freshly harvested, untreated mature pollen grain, *ca.* ×3 200. C. *In vitro*-germinated pollen tube, *ca.* ×6 700.

tional exine, coagulation and hydration commence from the germinal apertures. Where the exine is permeable to the acid, as in the case of pollen subjected to a pre-soak in sucrose media, coagulation also starts across the surface of the pollen grain. In pre-soaked pollen samples, the liberation of the pollen protoplast is prevented due to coagulation of peripheral cytoplasm across the

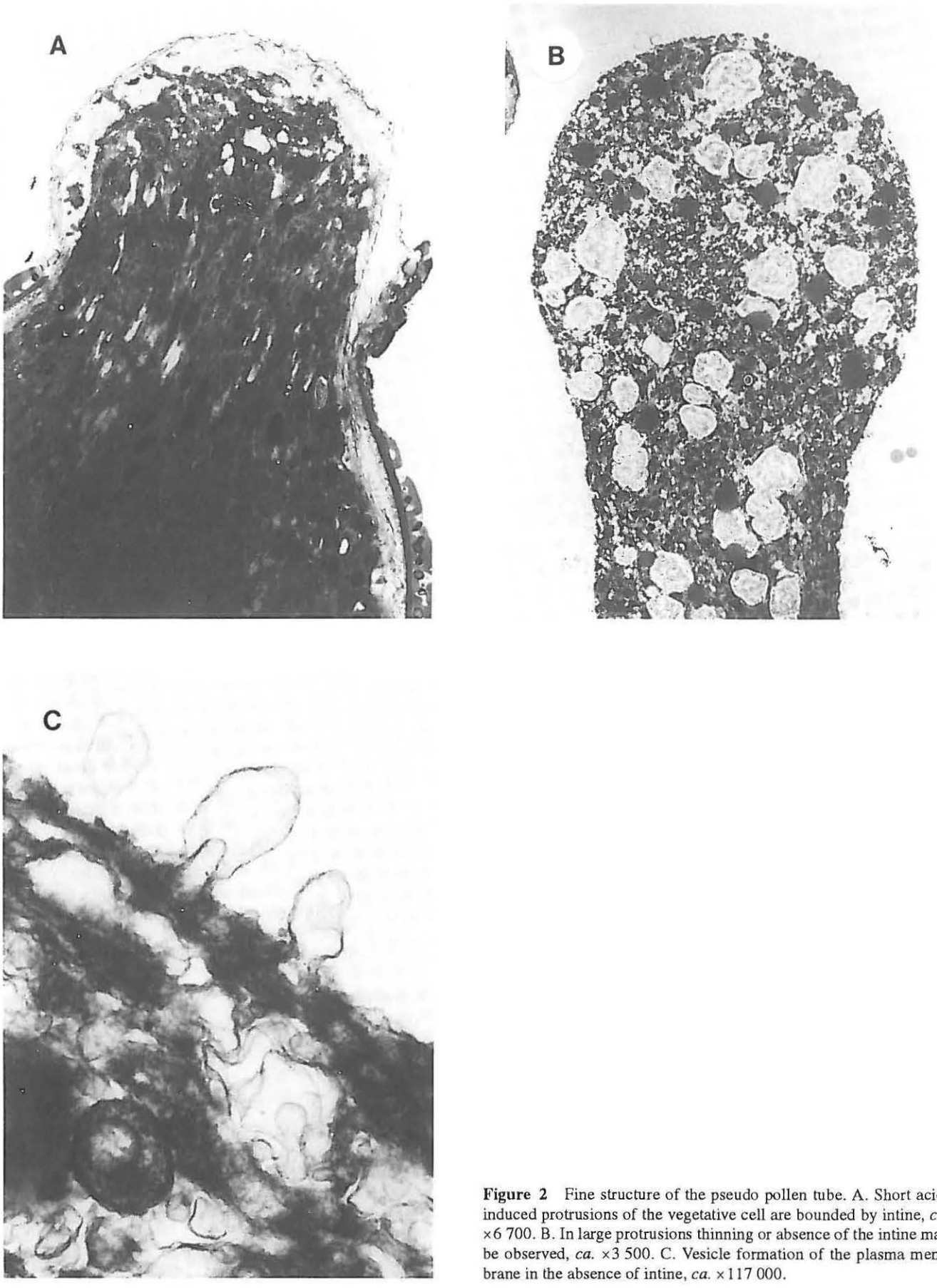

Figure 2 Fine structure of the pseudo pollen tube. A. Short acid-induced protrusions of the vegetative cell are bounded by intine, *ca.* ×6 700. B. In large protrusions thinning or absence of the intine may be observed, *ca.* ×3 500. C. Vesicle formation of the plasma membrane in the absence of intine, *ca.* ×117 000.

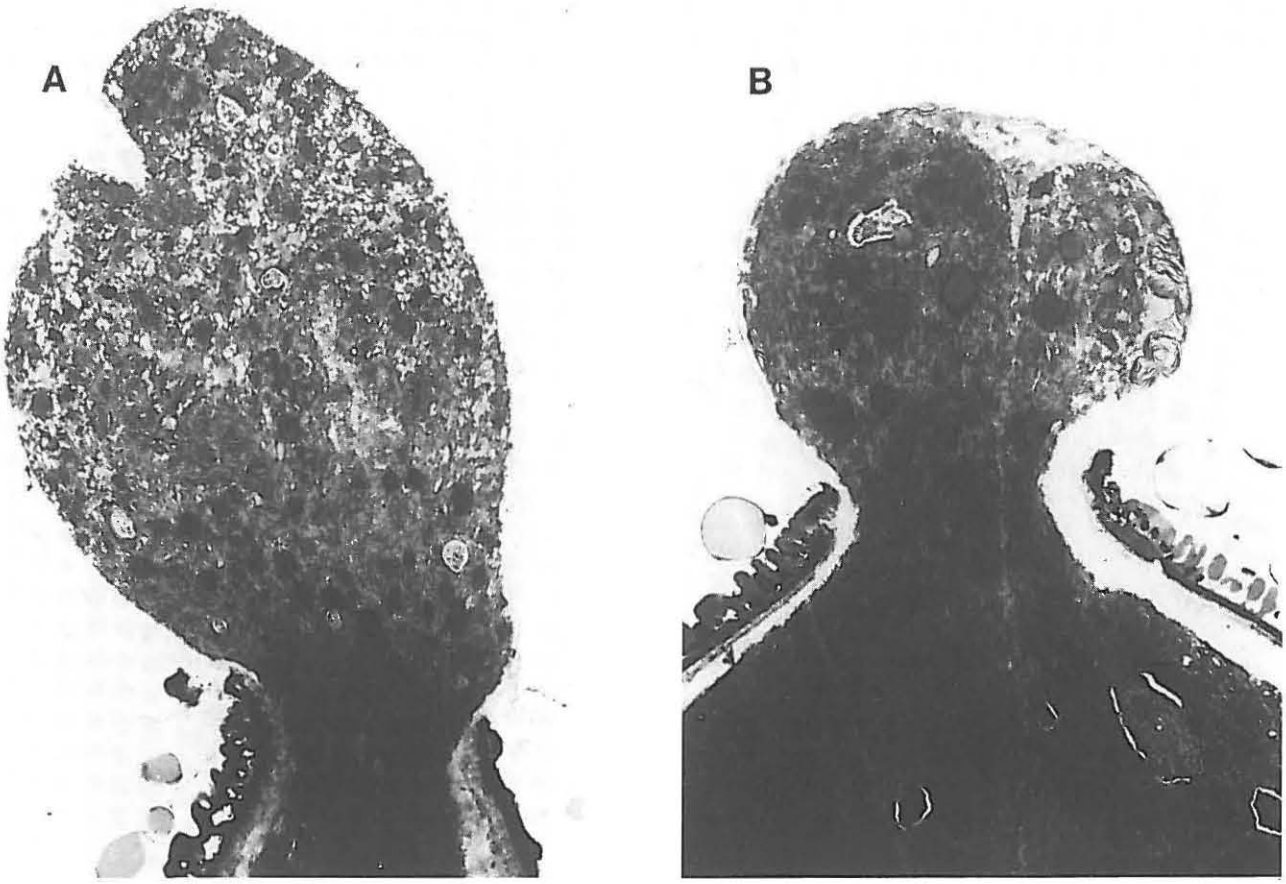

Figure 3 Bifurcation of the pseudo pollen tube tip, *ca.* ×2 900 (A), appears to form a funnel upon contact with the acid, *ca.* ×5 600 (B).

surface of the vegetative cell, resulting in a rigid protoplast which cannot be extruded via the germ pore.

Conclusion

It has been proposed that turgescence of the vegetative cell is an absolute prerequisite for the germination of pollen (Shivanna & Heslop-Harrison 1981). A cell with a ruptured or similarly impaired cell membrane cannot become turgid. Without an intact cell membrane, pollen grains suspended in acidic solution cannot swell and explode or form protrusions of the vegetative cell. The inorganic acid test of pollen viability determines the presence of the pollen protoplast, enclosed in an intact membrane. A pollen grain without a membrane-enclosed protoplast cannot fulfil its functional objective, i.e. to fertilize an egg cell. The inorganic acid test of pollen viability measures this quality of pollen and may provide a more effective viability testing procedure than cytoplasmic staining. As with cytoplasmic staining, the procedure is not suited to viability determinations in stored pollen, as loss of viability and vigour during storage cannot be measured. The test may provide a useful procedure for the estimation of pollen viability under field conditions, particularly to distinguish between sterile, partially sterile and fertile plants. Technically, this is the simplest of all pollen testing procedures and the only requirement is a low-magnification light microscope. Several pollen types lend themselves to this testing procedure, particularly aperturate pollen grains. To determine the merit of this procedure in pollen quality assessment and to evaluate its standing amongst the pollen tests available to plant breeders, comparative studies with other pollen testing procedures, such as the fluorochromatic reaction (Heslop-Harrison and Heslop-Harrison 1970) and correlation with seed set must be undertaken.

The treatment of pollen grains with a mixture of sulphuric acid and acetic anhydride is used as a standard method to destroy the cytoplasmic contents of pollen grains and to prepare the pollen grain exine for morphological examination (Erdtman 1960). However, this study has shown that the direct application of specific concentrations of sulphuric acid to pollen grains does not result in total destruction of the cytoplasm and organelles, but coagulation of these structures occur. Further development of this procedure may offer a valuable tool for workers in other fields of study. This reliable procedure offers a simple method to release the male gametophyte from its encasement and, with further development, may facilitate the study of the organelles of the pollen protoplast and the internal structure of pollen. Viable protoplasts cannot be obtained with this procedure (Linskens & Mulleneers 1967), but the possibility of harvesting viable generative cells by rapid removal of the acid after extrusion requires further investigation.

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